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Isolation and Characterization of Bacteria Degrading Sumandak and South Angsi Oils

(Pemencilan dan Pencirian Bakteria Pendegradasi Minyak Sumandak dan South Angsi)

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ABSTRACT

Four species of bacteria, Acinetobacter lwoffii, Aeromonas hydrophila, Pseudomonas aeruginosa *and* Pseudomonas *putida, were isolated from soil contaminated with hydrocarbons and selected for the determination of growth requirements and the ability to degrade petroleum hydrocarbon. The bacteria were grown in mineral salt medium (MSM) supplemented with two types of crude oil, either Sumandak or South Angsi at 1% (v/v) concentration. The optimum pH for growth of* A. hydrophila *and* P. aeruginosa *was 6.5 when grown with Sumandak and South Angsi oil. For* A. lwoffii *and* P. putida *the optimum pH for growth with Sumandak and South Angsi oil was 6.5 or 7.0, respectively. The growth of* P. aeruginosa *was the highest in MSM when supplemented with 1% South Angsi oil and 0.5% tryptone at pH 6.5 while, in Sumandak oil the growth was the highest when yeast extract was added. Gas chromatography analysis showed that the South Angsi crude oil components of* C_{12} *to* C_{25} *were more extensively degraded by A. lwoffii after 24 h of incubation compared to the other bacteria over the same period.*

Keywords: Bacteria; biodegradation; crude oil; petroleum hydrocarbon

ABSTRAK

Empat spesies bakteria, iaitu Acinetobacter lwoffii, Aeromonas hydrophila, Pseudomonas aeruginosa *dan* Pseudomonas putida *telah dipencilkan daripada tanah terlumus dengan hidrokarbon dan dipilih untuk penentuan keperluan pertumbuhan dan keupayaan untuk mendegradasi hidrokarbon petroleum. Bakteria dihidupkan di dalam medium garam mineral (MSM) yang dibekalkan dengan dua jenis minyak mentah, sama ada Sumandak atau South Angsi pada kepekatan 1% (i/i). pH optimum bagi pertumbuhan pencilan* A. hydrophila *and* P. aeruginosa *ialah 6.5 apabila dihidupkan dengan minyak Sumandak dan South Angsi. Bagi* A. lwoffii *dan* P. putida *pH optimum untuk pertumbuhan dengan minyak Sumandak dan South Angsi masing-masing adalah 6.5 dan 7.0. Pertumbuhan* P. aeruginosa *adalah paling tinggi apabila dibekalkan dengan 1% minyak South Angsi dan 0.5% tripton pada pH 6.5 manakala dalam minyak Sumandak, pertumbuhan adalah tinggi apabila ditambah yis ekstrak. Analisis kromatografi gas menunjukkan komponen* C_{12} *sehingga* C_{25} *dalam minyak mentah South Angsi lebih banyak didegradasikan oleh A. lwoffii berbanding bakteria lain selepas 24 jam pengeraman.*

Kata kunci: Bakteria; biodegradasi; hidrokarbon petroleum; minyak mentah

INTRODUCTION

Oil spills are an inevitable consequence of using petroleum. Spills can occur at the oil refinery, during transportation, natural seeps or during routine maintenance of infrastructure. These oil spills devastate the soil, surface and ground water and alter the microbial population at the polluted sites. Biodegradation by microorganisms is more favorable than chemical treatment for dealing with oil pollution since the microbes modify crude oils in beneficial ways and the end products are environmentally safe to all living things. Bioremediation of waste materials, which contain hydrocarbons and their derivatives, is based on the ability of microorganisms to increase their biomass growing on these substrates and to degrade them to non-toxic products, such as H_2O and CO_2 (Toledo et al. 2006).

Petroleum hydrocarbon can be degraded by various microorganisms such as bacteria, fungi and yeast (Chaillan et al. 2004). Studies have shown that most potential bacteria for petroleum hydrocarbon degradation have been isolated from areas contaminated with oil (Chaerun et al. 2004). The application of bacterial isolates in degrading crude oil involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali 2001). Microbes are dependent on nutrients for survival and these nutrients will be used to synthesize enzymes for the breakdown of hydrocarbons. Although numerous studies have been conducted on microbial consortia and enrichment (Chaerun et al. 2004), however, there is no data available for the Sumandak and South Angsi indigenous degraders of bacterial isolates. For this reason, the isolation and characterization of bacterial

isolates that can be used for bioremediation of Sumandak and South Angsi oil was carried out.

The objective of this study was to isolate and optimize the growth conditions of bacterial isolates that showed high potential for petroleum degradation. The South Angsi oil was classified as light crude oil and Sumandak as heavy crude oil based on their gas chromatography profiles.

Materials and Methods

Sampling of Soils and Media

Soils contaminated with oil were collected from two oil refineries in Malaysia (OGT-S1 and PPBST-1). Two types of medium were used, mineral salt medium (MSM) (Zajic & Supplinson 1972) supplemented with 1% (v/v) crude oil and vitamin mix (Bochaez et al. 1995) and MSM supplemented with 1% (v/v) oil but lacking vitamin mix. The pH of the medium was adjusted to 6.5 with 1N HCl or 1M NaOH. Two types of crude oil were used; Angsi and Sumandak. The medium was sterilised at 121°C for 15 minutes then added with 1% (v/v) crude oil and 0.01% mycostatin as a fungal inhibitor.

Isolation of Bacteria

Ten grams of soil were mixed with 50 ml 0.85% normal saline and incubated at 30°C for 30 minutes in an orbital shaker to homogenize the mixture, and the pH of the soil was measured (Metter Toledo pH meter). The mixture was centrifuged at 10414 *g*, 4°C for 10 min. The pellet was mixed and serially diluted with normal saline. The diluted suspension was plated onto MSM oil agar with and without vitamin mix by the spread plate method for isolation of hydrocarbon degrading bacteria. For the total count of bacteria, the diluted suspension was plated onto nutrient agar.

In order to isolate the desired bacteria, enrichment culture was used. Briefly, 1 mL of soil mixture was inoculated into 50 mL of MSM containing 1% (v/v) crude oil. The flasks were incubated aerobically for 7 days. After incubation, an inoculation loop of culture was streaked onto MSM agar containing 1% crude oil and incubated at 37°C for 24 h. Microbial colonies were picked randomly and subculture by streaking onto MSM plates several times until pure colonies were obtained.

Identification of Isolated Bacteria

Bacteria were identified by their morphological characteristics based on the shape, size and motility of the cells as well as colony morphology on nutrient agar (NA) plates. All isolates were examined by Gram's staining reaction to differentiate between Gram positive and Gram negative bacteria. Physiological characteristics were determined based on several biochemical tests such as oxidase, catalase, motility, triple sugar iron (TSI), hydrogen sulphide (H_2S) and gas production, indole, methyl red, Simmon's citrate, and Voges Proskaeur. Furthermore, the identification of bacteria were also confirmed using Microbact 24E Identification System (Oxoid) for the Gram negative isolates.

Determination of Optimum pH for Bacterial Growth

Standardized inocula was prepared as described by Azmy & Hamzah (2007) and were diluted into 20 mL of mineral salt medium (MSM) in a 100 mL Erlenmeyer flask. Two types of crude oil, Sumandak and South Angsi were added separately into the MSM, each at 1% (v/v) concentration. The pH of each medium was adjusted to 6.0, 6.5, 7.0 or 7.5 with 1 M NaOH or 1N HCl. The cultures were incubated at 37°C for 18 - 24 h in an orbital shaker at a speed of 150 rpm. The cultures were serially diluted 10-fold using sterile normal saline solution, and the growth was estimated by plating the bacteria on NA by the spread plate method.

Determination of Nitrogen Sources for Bacterial Growth

Nitrogen sources such as ammonium nitrate (NH_4NO_3), sodium nitrate ($NaNO_3$), yeast extract, tryptone and L-glycine were used in this study. Each of these nitrogen sources was added to 20 mL of MSM broth at a concentration of 0.5% (w/v) at the optimum pH determined previously. One percent of Sumandak and South Angsi oil was added separately into the medium. The cultures were incubated for 24 h at 37°C in an orbital shaker operated at 150 rpm. The growth of bacteria was monitored by the spread plate method and the number of colonies was expressed as colony forming unit per milliliter ($CFUmL^{-1}$).

Degradation of Hydrocarbon

The bacteria were inoculated into 50 mL of MSM with 1% of crude oil in a 100 mL Erlenmeyer flask with addition of yeast extract or tryptone at pH 6.5 or 7.0. The bacteria were incubated in an orbital shaker at 37°C and 150 rpm for 24 and 48 h. Medium without the bacteria was used as a control. At the 24 h time point, the flask was removed and the medium was centrifuged at 10414 *g* for 15 minutes to pellet the bacteria. The residual oil in the supernatant was extracted with dichloromethane. The oil-solvent mixture was removed using a separator funnel. Then the solvent was removed by evaporation using a rotary evaporator at 37°C and the oil was concentrated to 0.5 mL. One microliter of the extractable crude oil was analyzed on a 30 m polydimethyl siloxane capillary column (HP 1) in a capillary gas chromatograph (Agilent-Auto System) equipped with a flame ionization detector (FID) using the HP ChemStation software. The oven temperature was initially set to 30°C and increased at a rate of $4°C\ min^{-1}$ to 300°C.

RESULTS AND DISCUSSION

ISOLATION AND IDENTIFICATION OF BACTERIA

Soil samples contaminated with oil have a different pH depending on the depth and location (Table 1). The pH of the soil from the surface to 50 cm below the surface was in the range of acidic (5.04 to 5.78) and at 60 cm the pH was 6.27. As the depth was increased, the value of pH also increased towards alkaline value. This result showed that soils contaminated with oil were acidic. The range of pH for soil samples at the Melaka oil refinery were 6.0 to 6.8 while soil contaminated with diesel and engine oil was from 5.4 to 6.4 (Ghazali et al. 2004). After processing the soil samples, the number of colonies decreased as sample depth increased, showing that most of the bacterial degrading crude oil were growing aerobically in the surface soil.

The hydrocarbon-degrading bacteria were obtained by culture enrichment technique with South Angsi and Sumandak as the sole carbon for energy. Upon isolation and selection, 4 isolates were chosen in this study as they produced good growth on MSM plates containing Sumandak and Angsi oils. The bacteria exhibited the ability to degrade Sumandak and Angsi oils by showing hydrolysis zones on MSM plates containing the oils after 5 days of incubation. The organisms were identified by morphological and biochemical techniques using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The bacteria were identified as *Pseudomonas aeruginosa, Pseudomonas putida, Acinetobacter lwoffii* and *Aeromonas hydrophila*.

All of these bacteria were Gram-negative rod, motile, oxidase and catalase positive except the *A. lwoffii* showed catalase negative and non motile organism. Some of the carbon sources such as glucose, mannitol, sucrose, arabinose and xylose were fermented by *A. hydrophila*. All the isolates are not sulphide reducer and without CO_2 production (Table 2).

Pseudomonas is a common bacterium capable of degrading hydrocarbons (Das & Mukherjee 2007). Therefore, it is not uncommon for a number of *Pseudomonas* strains capable of degrading petroleum hydrocarbon should be isolated from areas receiving petroleum waste discharges. In addition, *Pseudomonas aeruginosa* was found in a consortium of bacteria from sandy and loamy soils that can degrade hydrocarbons in light fuel oil (Hawle-Ambrosch et al. 2007). It was also shown that *Pseudomonas aeruginosa* (ATCC 27853), which is a clinical

TABLE 1. pH and colony count of bacteria (cfu/mL) grown in MSM supplemented with 1% crude oil for 2 weeks incubation from different locations and depths

Location	Depth	pH	Types of crude oil	
			Sumandak	Angsi
OGT-S1	0 cm	5.04	1.73×10^8	7.4×10^7
	10 cm	5.13	5.6×10^7	2.6×10^7
	20 cm	5.26	2.0×10^7	1.1×10^7
	30 cm	5.33	1.9×10^7	3.7×10^6
	40 cm	5.56	4.5×10^6	5.1×10^6
	50 cm	5.78	9.9×10^6	6.3×10^6
	60 cm	6.27	9.3×10^6	4.9×10^6
PBST-1	20 cm	6.31	1.4×10^7	3.1×10^6
	40 cm	6.46	3.5×10^6	3.3×10^6

TABLE 2. Characteristics of isolates and identification results

Isolates	Preferred Organism	Biochemical tests									
		TSI		Mot	Oxi	Cat	Ind	Gas	H_2S	Cit	MR	VP
		s	b									
A05	*Pseudomonas putida*	r	r	+	+	+	-	-	-	+	+	-
A10	*Aeromonas hydrophila*	r	r	+	+	+	-	-	-	-	+	-
A30	*Pseudomonas aeruginosa*	r	y	+	+	+	-	-	-	+	+	-
B37	*Acinetobacter lwoffii*	r		-	+	-	-	-	-	+	-	+

Oxi = oxidase, Cat = catalase, Mot = motility, Ind = indole, TSI = triple sugar iron agar, s = slant, b = butt, r = red (alkaline), y = yellow (acidic), gas = production of CO_2 gas, H_2S = production of H_2S, cit = Simmon's citrate, MR = methyl red, VP = Voges Proskaeur, + = positive, - = negative

strain, can grow on hydrocarbon as sole carbon source and is a good degrader of oil (Szoboszlay et al. 2003). Similarly, *Pseudomonas putida* is able to degrade hydrocarbons such as benzene (Munoz et al. 2007), toluene, *p*-xylene (Yu et al. 2001), biphenyl (Ohta et al. 2001) and phenol (Juang & Tsai 2006). Encountering *Pseudomonas* in oil-contaminated soil is not rare, however we believe this is the first report about the ability of *Pseudomonas aeruginosa* and *Pseudomonas putida* to use Sumandak and Angsi crude oils as a carbon source.

This study also showed that *Acinetobacter lwoffi* was able to grow in both types of oil. A study by Breuil et al. (1978) showed *Acinetobacter lwoffi* strain O_{16}, a facultative psychrophile, can grow on crude oil, hexadecane, octadecane, and most alkanes. Adebusoye et al. (2006) also showed *A. lwofii* and *P. aeruginasa* had isolated these bacteria from tropical stream and they are capable of degrading of pure hydrocarbons including cycloalkane and aromatic hydrocarbon.

Previously, only *Acinetobacter baumannii* has been reported to assimilate saturated and aromatic hydrocarbons (Chaineau et al. 1999). This *Acinetobacter* sp. was isolated from an agriculture soil sample in France. *Acinetobacter* is not usually identified as a bacterium with the ability to degrade hydrocarbons. However, the bioemulsifier produced by *Acinetobacter* is used to accelerate the degradation of hydrocarbon (Barkay et al. 1999). Therefore, based on the ability to grow on both of the oils, isolate *Acinetobacter lwoffi* might have the potential to carry out biodegradation of hydrocarbon pollutants.

A. hydrophilia was also able to grow in both South Angsi and Sumandak oils as sole carbon source, indicating that the bacterium also has the potential to degrade hydrocarbons. Ajisebutu (1988) showed two species of *Aeromonas* capable of degrading 5 types of crude oils in the presence of 0.5% (w/v) NaCl.

THE EFFECTS OF pH ON THE GROWTH OF ISOLATED BACTERIA METABOLIZING SUMANDAK AND ANGSI OIL

All of the bacteria were able to grow at different rates depending on the type of oil used in the medium. In MSM supplemented with Sumandak oil, *P. aeruginosa, P. putida* and *A. lwoffii* showed the highest growth at pH 6.5, while *A. hydrophila* preferred to grow at pH 7.0 (Figure 1). In MSM supplemented with South Angsi oil, *P. aeruginosa* and *A. lwoffii* showed the highest growth at pH 6.5 while *P. putida* and *A. hydrophila* showed maximum growth at pH 7.0 (Figure 2). Hence, the optimum pH for the growth of *P. aeruginosa* and *P. putida* was 6.5 independent of the type of oil used. In contrast, *A. hydrophila* and *A. lwoffii* were dependent on the type of oil used. However, the differences of bacterial growth *A. hydrophila* and *A. lwoffii* in both oils at pH 6.5 and 7.0 were less than 10×10^6 cfumL^{-1}, which was considered very small. This indicated that either pH 6.5 or 7.0 can be used to study the growth of *A. hydrophila* and *A. lwoffii*. Based on this finding, the pH used for biodegradation of hydrocarbon by *P. aeruginosa* and *P. putida* was 7.0, whereas the pH for *A. hydrophila* and *A. lwoffii* can be either 6.5 or 7.0 depending on the type of crude oil used.

Yuan et al. (2002) isolated *Pseudomonas fluorescens* and *Haemophilus* sp. from soil contaminated with petrochemical effluent discharge. These bacteria were able to degrade polyaromatic hydrocarbons (PAH) and grew at an optimum temperature of 30°C and pH 7.0. Other studies also showed an optimal pH of 7.0 for the maximum growth (Mishra et al. 2001) and degradation of crude oils by individual and mixed bacterial consortium (Sathishkumar et al. 2008).

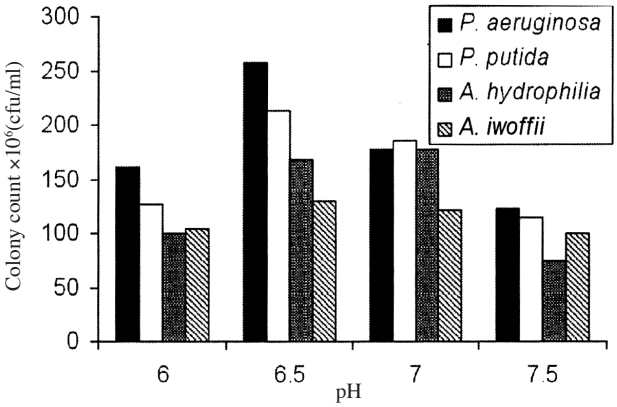

FIGURE 1. The effect of pH on the bacterial growth in mineral salt medium supplemented with Sumandak oil (1%, v/v)

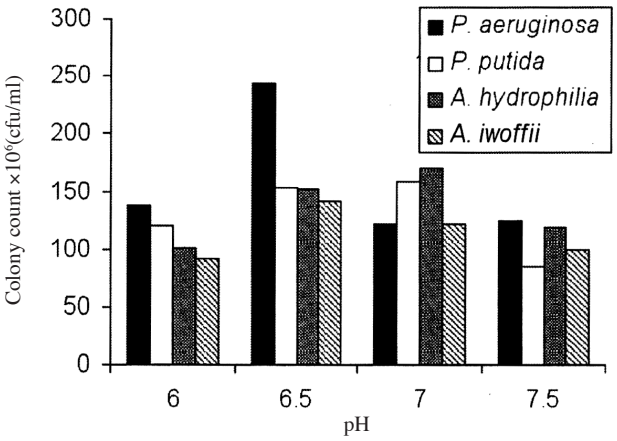

FIGURE 2. The effect of pH on the bacterial growth in mineral salt medium supplemented with South Angsi oil (1%, v/v)

THE EFFECT OF NITROGEN SOURCES ON THE GROWTH OF ISOLATED BACTERIA

The bacteria were grown in MSM supplemented with Sumandak oil and different nitrogen sources at 0.5% concentration. Out of four nitrogen sources used in this experiment, only the addition of yeast extract and tryptone showed a significant increase in growth of the isolates. These results showed that *P. aeruginosa* and *A.*

lwoffii had the highest growth with the addition of yeast extract, while *P. putida* and *A. hydrophila* grew optimally in tryptone (Figure 3). When South Angsi oil was used together with MSM, the growth of *P. aeruginosa, P. putida* and *A. lwoffii* was the highest in the presence of tryptone. Meanwhile, for *A. hydrophila* the growth was the highest in yeast extract (Figure 4). When glycine and ammonium nitrate were used in the MSM growth media, the growth of bacteria did not increase significantly.

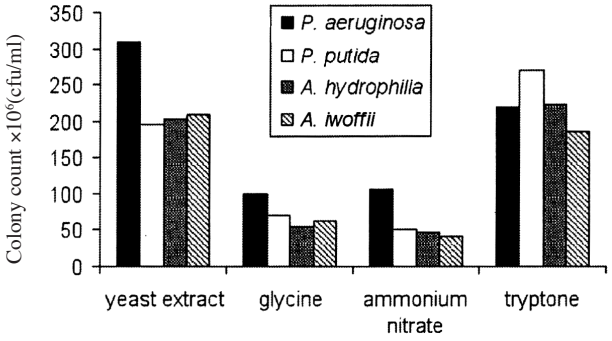

FIGURE 3. The effect of different nitrogen sources on the bacterial growth in mineral salt medium supplemented with Sumandak oil (1%, v/v)

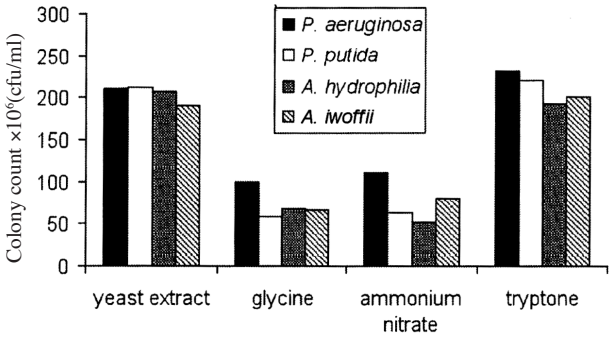

FIGURE 4. The effect of different nitrogen sources on the bacteria growth in mineral salt medium supplemented with South Angsi oil (1%, v/v)

Yeast extract was shown to enhance the biodegradation of phenanthrene in soil by consortia of microbes (Yuan et al. 2000; Chang et al. 2001). However, addition of potassium nitrate delayed the degradation of phenanthrene (Yuan et al. 2000). The same phenomenon may have occurred in this study, as addition of yeast extract contributed significantly to growth. However, there is evidence that *Pseudomonas fluorescens* and *Haemophilus* sp. were able to use nitrate, ammonia, sulfate and phosphate to support their growth during PAH degradation in soil (Yuan et al. 2002). Based on these results, either yeast extract or tryptone was used for determination of hydrocarbon degradation.

DEGRADATION OF HYDROCARBON

The end products of hydrocarbon degradation by the bacteria were analyzed by gas chromatography. The bacteria were grown in MSM with the addition of 0.5% yeast extract and 1% South Angsi oil. *P. aeruginosa* reduced the hydrocarbon components from C_{12} to C_{30} more efficiently than *A. lwoffii* (Table 3). Gas chromatography of crude South Angsi oil degradation products showed *P. aeruginosa* degraded 48% of total petroleum hydrocarbons (TPH) after 24 h and 77% after 48 h of incubation (Table 4). Meanwhile, isolate *A. lwoffii* degraded only 28% and 62% of TPH after 24 and 48 h of incubations, respectively (Figure 5). From the percentage of degradation for C_{12} to C_{20}, *A. lwoffii* appears to degrade the C_{11} to C_{17} fractions more effectively than *P. aeruginosa* after 48 hours of incubation. The C_8 to C_{10} fractions were probably lost to evaporation during 48 hours of incubation. *P. putida* was the least effective in degrading the oil components compared to the other bacteria. A further incubation for 48 hours, it only reduced the hydrocarbon content by an additional 10% (results not shown).

The chromatograms showed *P. aeruginosa* metabolized the oil effectively due to the decreasing size of the peak from C_{12} to C_{30}. Carbon 12 to carbon 20 is defined as aromatic hydrocarbon, and is associated with the release of petroleum products into the environment. Each type of crude oil has its own characteristic composition. Microbial degradation was affected by the composition of the oil, with

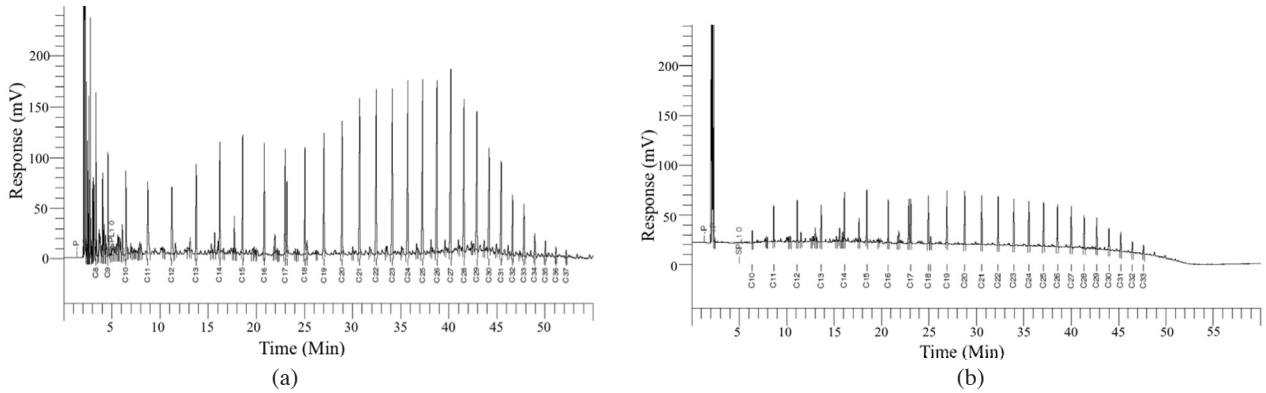

FIGURE 5. Capillary gas chromatography analysis of South Angsi oil; (a): Control MSM + 0.5% tryptone at pH 6.5 without bacteria; (b): *P. aeruginosa* was grown in MSM + 0.5% tryptone at pH 6.5 after 48h incubation.

TABLE 3. Percentage degradation of South Angsi crude oil for n12-n20 after 48 hours incubation

Isolate	n –Cn (%)								
	12	13	14	15	16	17	18	19	20
P. aeruginosa	65.0	68.0	60.0	63.6	63.6	57.9	61.5	64.4	66.9
A. lwoffii	81.5	66.5	64.0	65.3	65.3	75.7	55.4	50.6	51.8

some oils being degraded more completely than others. *P. aeruginosa* and *A. lwoffii* attacked the long chain n-alkanes (C_{12} to C_{20}), possibly due to their less toxic nature. Short chain alkanes are toxic for many microorganisms.

When the same set of experiments was performed using Sumandak oil, degradation was more efficient. Sumandak oil is categorized as light oil, while South Angsi is heavy oil. The results showed that after 24 hours and 48 hours of incubations, *P. aeruginosa* degraded the Sumandak oil components most efficiently followed by *A. lwoffii*, while *A. hydrophila* and *P. putida,* were the least efficient (Table 4). After 48 hours of incubation, *P. aeruginosa* and *A. lwoffii* showed a further 50% degradation of the oil (Figure 6) while *P. putida* and *A. hydrophila* did not showed any further change in degradation (data not shown). The selective features of these isolates are important in the formation of consortia for complete oil degradation.

Pristane, phytane and hopane can be used as biomarkers for degradation since the degradation of these compounds at much slower rate than the straight-chain alkanes (Balba et al. 1998). Reduction of pristane peak by *P. aeruginosa* was the highest followed by *A. lwoffii, A. hydrophila* and *P. putida* after 48 hours cultivation in MSM added with 1% South Angsi and Sumandak oil (Table 5). Most importantly, these chromatographic characteristics correspond to criteria used to quantify petroleum degradation due to microbial activities.

CONCLUSION

This study showed that four species of bacteria, *Acinetobacter lwoffi, Aeromonas hydrophila, Pseudomonas aeruginosa* and *Pseudomonas putida*, isolated from contaminated soil were able to degrade Sumandak and South Angsi oil. They showed optimum growth at pH 6.5 or 7.0 and better growth with the addition of yeast extract or tryptone. *Pseudomonas aeruginosa* was able to degrade both Sumandak and South Angsi oils at highest percentage compared to other isolates.

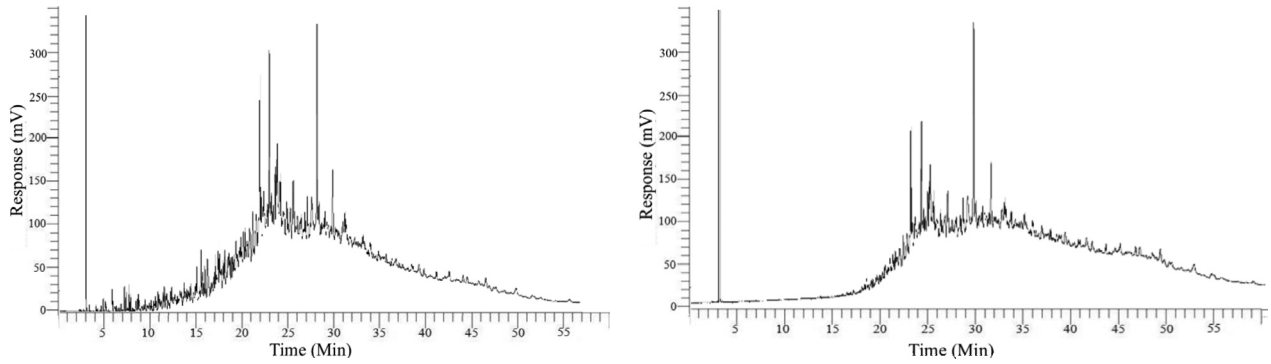

FIGURE 6. Capillary gas chromatography analysis of Sumandak oil (a) Control MSM + 0.5% tryptone at pH 6.5 without bacteria. (b) *P. aeruginosa* was grown in MSM + 0.5% tryptone at pH 6.5 and incubated for 48 h

TABLE 4. Percentage of TPH degradation of South Angsi and Sumandak crude oil by *A.lwoffii* and *P. aeruginosa*

	Percentage degradation of TPH			
	South Angsi		Sumandak	
	24 h	48 h	24 h	48 h
P. aeruginosa	48	77	25	59
A. lwofii	28	62	13	51

TABLE 5. Percentage degradation of pristane after 48 hours incubation

	Percentage degradation of pristane			
	P. aeruginosa	*P. putida*	*A.lwoffii*	*A. hydrophila*
South Angsi	36.4	6.2	25.4	9.71
Sumandak	45	8.39	32.9	11.45

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